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Determination of the levels of catechol estrogens (CE) in breast tissue constitutes important evidence for the hypothesis that human breast cancer and certain other cancers are initiated by activation of CE to CE-3,4-quinones (CE-3,4-Q), which form depurinating DNA adducts. Apurinic sites generated by loss of the depurinating adducts can produce oncogenic mutations. Because only nonmethylated CE can be oxidized to CE-Q, the ratio of nonmethylated *vs* methylated CE should be higher and/or the level of catechol-O-methyltransferase (COMT) in breast tissue lower in women with breast cancer compared to women without disease. Our assays focus particularly on the 4-hydroxyestrogens and 4-hydroxy COMT. We are developing an assay to determine the levels of CE in breast tissue from pre- and post-menopausal women with and without breast cancer by gas chromatography/mass spectrometry (GC/MS) analysis. The GC/MS methods have been developed, and we are working to optimize the extraction of CE from mammary tissue. At the end of this grant we expect to have collected sufficient data on the levels of CE in breast tissue to make preliminary comparisons of women with and without breast cancer.

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Eleanor H. Rogan 6/4/98

PI - Signature Date

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INTRODUCTION

The role of estrogens on the induction of breast cancer can be related to initiation, promotion, or both. We hypothesize that the initiating event for human breast cancer and certain other human cancers is associated with activation of endogenous catechol estrogens (CE), which are hydroxylated metabolites of estrone (E_1) and 17β-estradiol (E_2), 2-OHE_1(E_2) and 4-OHE_1(E_2) [1].

In mammalian cells, CE are predominantly conjugated to their 2-, 3- or 4- monomethoxy derivatives by catechol-O-methyltransferases (COMT) [2]. These enzymes are considered to be protective enzymes because only nonmethylated CE can be oxidized to their quinones (CE-Q) by peroxidases and cytochrome P-450 (Fig. 1). In our hypothesis, CE-3,4-Q are the ultimate carcinogenic forms of estrogens because these electrophiles can covalently bind to the nucleophilic groups of DNA to form depurinating adducts that could initiate cancer by mutating critical genes [1]. Depurinating adducts are lost from DNA by hydrolysis of the glycosidic bond, leaving apurinic sites, which if not repaired, could be mis-replicated to produce oncogenic mutations.

Because only nonmethylated CE can be oxidized to CE-Q, the ratio of nonmethylated *vs* methylated CE should be higher in women with breast cancer compared to women without disease. We expect to find that the ratio of nonmethylated *vs* methylated CE is higher in women with breast cancer and/or the level of COMT in breast tissue is lower. This approach presumes that the levels of nonmethylated *vs* methylated CE and COMT are characteristic of a woman and, thus, the results obtained with breast cancer patients are similar to those that would have been obtained before development of the tumor.

In this project, we are determining the levels of CE and COMT in breast tissue from pre- and post-menopausal women with and without breast cancer. CE are quantified by gas chromatography/mass spectrometry (GC/MS) analysis after extraction from tissue. COMT activity will be quantified by an assay using [methyl-^{3}H]S-adenosyl methionine. Our assays will focus primarily on the 4-hydroxyestrogens, 4-OHE_1 and 4-OHE_2, and their methylation by 4-hydroxy COMT. At the end of this project we expect to have collected sufficient data on the levels of CE and COMT in breast tissue to make preliminary comparisons of women with and without breast cancer.

The results of the proposed studies are expected to provide the basis for very early **detection** of women at risk for developing breast cancer and to lay the foundation for developing approaches to **preventing** manifestation of this disease. In addition, new information concerning the **etiology** of breast cancer will be obtained.

BODY

Assumptions

We hypothesize that the initiating event for human breast cancer and certain other human cancers is associated with activation of endogenous CE, which are metabolites of E_1 and E_2.

E_1 and E_2, which are continuously interconverted (Fig. 1), are metabolized via two major pathways: hydroxylation at the 16α position and hydroxylation at the 2 or 4 position [2]. The latter pathway produces CE. In mammalian cells, CE are predominantly conjugated to their 2-, 3- or 4-monomethoxy derivatives by COMT (Fig. 1) [2]. These enzymes are considered to be protective enzymes because only nonmethylated CE can be oxidized to their quinones by peroxidases and cytochrome P-450 [1,3-6]. In our hypothesis, CE-Q are the ultimate carcinogenic forms of estrogens because these electrophiles can covalently bind to the nucleophilic groups of DNA to form adducts. Furthermore, redox cycling generated by reduction of CE-Q to CE-semiquinones and subsequent oxidation back to CE-Q can generate hydroxyl radicals that cause additional DNA damage, as proposed by Liehr and Roy [7] and Nutter, *et al.* [8].

The adducts formed by reaction of CE-Q with DNA could initiate cancer by mutating critical genes [1]. Evidence indicates that the depurinating adducts, which are lost from DNA by hydrolysis of the glycosidic bond to leave apurinic sites, play the major role in tumor initiation [9,10].

Rapid O-methylation of CE, catalyzed by COMT, is thought normally to convert these catechols to inactive O-methyl ethers, thus removing the CE from possible oxidation to CE-Q. If the formation of CE exceeds the capacity of a cell to catalyze O-methylation, an accumulation of CE might ensue [11,12]. Thus, determination of the levels of COMT, particularly 4-OH COMT, in this tissue may show that the level of this enzyme is lower in tissues from susceptible women.

An integral part of our hypothesis is that a high level of nonmethylated *vs* methylated CE and/or low level of COMT is a persistent characteristic of a woman susceptible to breast cancer. Thus, by analyzing breast tissue from patients we expect to find results similar to those that would have been found prior to tumor induction. It is necessary to conduct these analyses with breast tissue because only the tissue susceptible to the tumor will presumably reflect anomalies. Technology for determination of CE in breast tissue has been reported [13], but we have not been able to use these methods with success, and we consider the levels of CE reported questionable. Therefore, we are developing new procedures to obtain the desired specificity and sensitivity.

We have established an interdisciplinary group to investigate the hypothesis that activated CE are initiators of human breast cancer. Dr. James Edney is the surgeon who selects patients and removes the breast tissue samples. Dr. Sonny Johansson is responsible for sampling and analyzing the tissues histopathologically. Dr. Ercole Cavalieri is responsible for supervising synthesis of CE and derivatives, extraction of the samples and isolation of CE by HPLC. Dr. Ronald Cerny is responsible for optimizing existing methodology for gas chromatography/mass

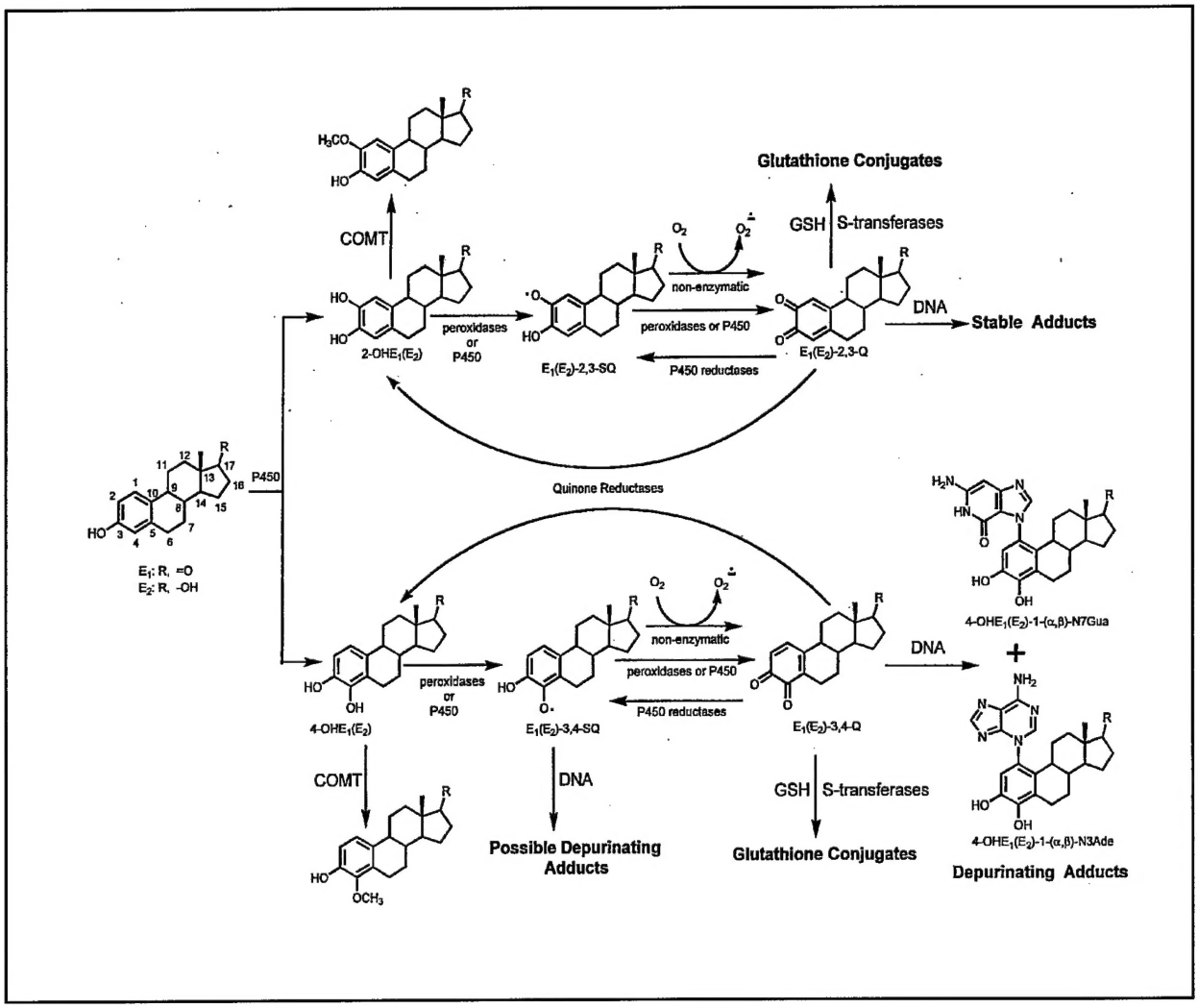


Figure 1. Metabolism of E_1 to CE, CE-Q, monomethoxyCE and the depurinating DNA adducts 4-OHE_1-(1&2)-N7Gua.

spectrometric (GC/MS) analysis of CE and carrying out these assays. Dr. Eleanor Rogan is responsible for supervising analysis of COMT by the [methyl-^{3}H]S-adenosyl methionine assay. Dr. James Anderson will statistically evaluate the data from analyses of CE and COMT.

Experimental Methods

1. Experiments to accomplish Specific Aim #1: Determine the levels of CE in breast tissue specimens from women with and without breast cancer

Covalent binding of activated CE to DNA leads to DNA damage that could initiate the cancer process [1]. With elevated rates of CE synthesis and/or deficient protective methylation of CE by COMT, the CE can be oxidized to CE-Q by peroxidases and/or cytochrome P-450. To test

this hypothesis, we plan to analyze specimens of "normal" breast tissue from women with and without breast cancer to determine the levels of CE and their monomethoxy derivatives, focussing particularly on the 4-OH-CE. We recognize that hormonal changes occur at menopause; thus, we will analyze separately the two cohorts, pre- and post-menopausal, of women with and without breast cancer. A basic premise of this study is that a high level of nonmethylated *vs* methylated CE and/or low level of COMT is a persistent characteristic of a woman susceptible to breast cancer. Thus, by analyzing breast tissue from patients, we expect to find results similar to those that would have been found prior to tumor induction.

a. Study subjects. Female patients available for study are those scheduled for breast biopsy because of suspected breast cancer or other breast disease. It is expected that this group of patients at the University of Nebraska Medical Center will include about 60 patients per year that will be diagnosed with breast cancer as a result of the biopsy; an equal number of control patients will also be available. Ca. 25-33% of the breast cancer patients will be premenopausal.

Women with and without breast cancer are selected by Dr. Edney and asked to contribute specimens prior to surgery. During surgery, specimens weighing at least 1 g are cut from histopathological samples by Dr. Johansson and frozen at -80 °C. Dr. Johansson establishes the histopathological diagnosis of the tissue (i.e., normal tissue or breast carcinoma).

b. Determination of CE and monomethoxyCE by GC/MS. Combination of the high separation efficiency of capillary GC with the specificity of MS is well established for trace analyses of complex mixtures. GC/MS has been used to analyze some CE from rat microsomes [14] and rat intestinal mitochondria [15], and for quantitation of CE in human breast tissue and cyst fluid (although these results appear questionable) [13]. The combined method has also been used to measure the levels of E_2-17-fatty acid esters in tissues [16]. In these studies, the CE were analyzed as trimethylsilyl (TMS) derivatives to make them more amenable for GC. Another derivatization process is to form heptafluorobutyl esters [17]. Each of these studies reports the analysis of only a few of the expected metabolic forms of E_1 and E_2. Although one study provides retention times and representative mass spectra for 14 of the expected metabolites or precursors, the tissue levels of only three CE were determined [13]. The detection limits for the TMS derivatives were in the ng range [13]. The use of a high resolution magnetic sector instrument in a selected ion monitoring (SIM) mode lowers the detection limit into the pg range [14].

To test accurately our hypothesis that the relative levels of CE in breast tissue may serve as a "marker" for the risk of cancer, we are developing an analytical protocol that quantifies as many of the E_1 and E_2 metabolites as possible. The methodology must account for the wide range in the amounts of the various target analytes, from μg/g tissue of E_1 or E_2 to pg/g tissue of CE. The procedure is being validated through both duplicate extractions and duplicate GC/MS analyses.

Rat mammary tissue spiked with representative CE was originally used in experiments to establish the analytical method. The large amount of fat in mammary tissue complicated our

efforts, and we decided to use hamster kidney (a target tissue for carcinogenesis by CE [18, 19]). Tissue is weighed, minced into small pieces with a razor, ground into a fine powder in liquid nitrogen and split into two equal fractions. One fraction is then suspended in 50 mM ammonium acetate, pH 4.0 (containing 1 mg/ml ascorbic acid)/methanol/dimethylformamide (5:5:1), and extracted twice with equal volume of hexane. The solid residue is centrifuged off and the supernatant is applied to a tC18 Sep-Pak cartridge. The cartridge is washed with successively increasing amounts of acetonitrile in 50 mM ammonium acetate, pH 4.0 (with 1 mg/ml ascorbic acid). Fractions are collected, concentrated and analyzed by HPLC.

HPLC is conducted on a reverse-phase Luna C18 column with detection by an ESA CoulArray multichannel electrochemical detector. Adducts are eluted with a linear 45 min gradient, starting with acetonitrile/methanol/water/1 M ammonium acetate, pH 4.4 (15:10:65:10), and ending with acetonitrile/methanol/water/1 M ammonium acetate, pH 4.4 (30:10:50:10), at a flow rate of 1 mL/min. The eight electrochemical cells in the detector are set at potentials between 0 and 560 mV. Peaks are identified by retention time and peak ratios between channels in the electrochemical profile.

The other fraction is derivatized in N,O-bis(trimethylsilyl)tri-fluoroacetamide (BSTFA) and analyzed by GC/MS at mass resolution of 10,000, monitoring the molecular ion of each derivatized compound. A DB-5 capillary column (30 m x 0.32 mm, 0.32 μ) is used and data are acquired on a Kratos MS-50 double-focusing mass spectrometer/Kratos-Mach 3 data system. The high resolution mass spectrometry operated in mass profiles mode, combined with the capillary GC separation, provides good method specificity. Deuterated analogues of the estrogen metabolites are used as internal standards. The procedure detection limit is in the sub-nanogram range with excellent accuracy and precision. We propose to quantify the levels of twelve available analytes: E_1 and E_2; 2-OH-, 4-OH-, and 16α-OH- E_1 and E_2; and 2-OCH_3- and 4-OCH_3- E_1 and E_2.

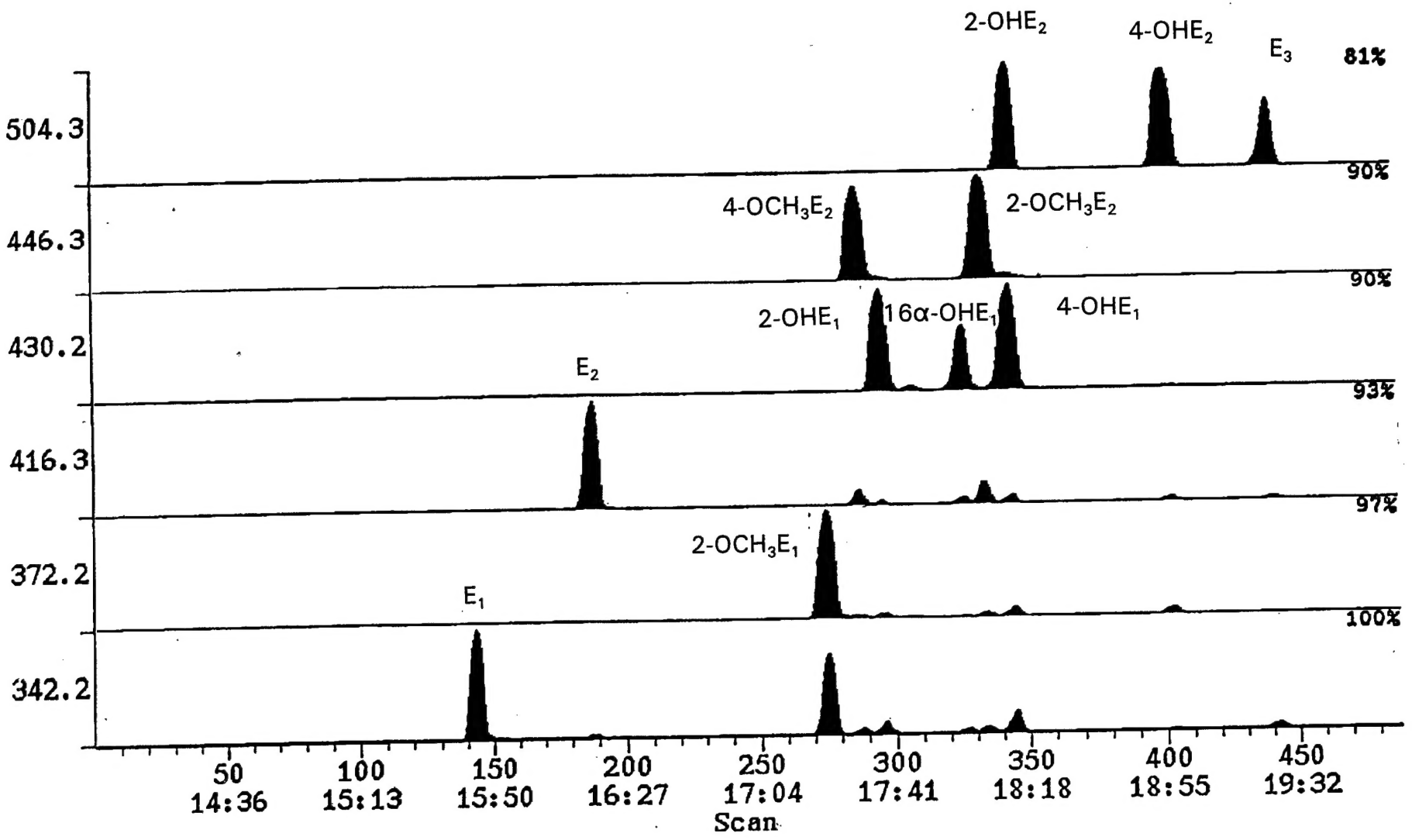
2. Experiments to accomplish Specific Aim #2: Determine the levels of COMT in breast tissue specimens from women with and without breast cancer

Covalent binding of activated CE to DNA leads to DNA damage that could initiate the cancer process. With elevated rates of CE synthesis and/or deficient protective methylation of CE by COMT, the CE can be oxidized to CE-Q by peroxidases and/or cytochrome P-450. As part of testing this hypothesis, we plan to analyze specimens of "normal" breast tissue from women with and without breast cancer to determine the level of cytosolic COMT activity. The samples will be from the tissues collected as described above, but we will begin this work during Year 3 of this project. Alternatively, we may decide to measure COMT by using a recently described RFLP method [20].

Results and Discussion

The GC/MS methods have been tested and found to detect E_1, E_2, their CE and monomethylCE (Fig. 2). Originally, we hoped to be able to analyze the CE metabolites after homogenizing the breast tissue. This did not prove to be successful, especially since the levels of

Figure 2.GC/MS of CE Metabolites



the CE metabolites are several orders of magnitude lower than those of the parent estrogens, E_1 and E_2. So we began to develop an effective extraction procedure. The extraction procedure described above has not proven to extract the CE metabolites as effectively as desired. In the first analysis of human breast tissue samples, 4-OHE_2 and 2-methoxyE_2 were tentatively identified. We then analyzed a breast sample spiked with CE metabolites and extracted with 70% chloroform/20% dimethylformamide/10% acetic acid, and concentrated on a C-18 Sep-Pak cartridge. No adducts were observed by GC/MS, although standards at the same levels were observed. This suggested that some interfering materials are present in the tissue specimen and further development of extraction procedures was necessary.

The extraction procedure described in the Experimental Methods section above has been worked out to separate CE metabolites and adducts with validation by HPLC with electrochemical detection. The HPLC method separates CE metabolites and derivatives (Fig. 3 and 4), and the CE derivatives are detected in hamster kidney tissue spiked with CE metabolites and derivatives (Fig. 5).

At present we are analyzing by HPLC and GC/MS tissue samples spiked with CE metabolites, but results are not yet available. We hope these methods are now successful.

CONCLUSIONS

Several experimental facts support the hypothesis that CE-3,4-Q react with DNA to form N7Gua and N3Ade depurinating adducts, leaving apurinic sites in the DNA, which, if not repaired, can generate mutations that initiate breast cancer. First, 4-$OHE_1(E_2)$ are carcinogenic in the kidney of Syrian golden hamsters, whereas the 2-$OHE_1(E_2)$ are not [18, 19]. Second, E_1-3,4-Q induced hepatocarcinomas in young male $B6C3F_1$ mice [1]. Third, after treatment of rat mammary gland *in vivo* with 4-$OHE_1(E_2)$ or the corresponding CE-3,4-Q, the depurinating DNA adduct, 4-$OHE_1(E_2)$-1-N7Gua, has been isolated from the mammary tissue (The N3Ade adduct standard was not available when this work was conducted) [1]. These findings provide an underpinning for this research project. Our analytical method for CE and methylCE has been developed. Once we are satisfied that our procedures for extracting CE metabolites from tissue are optimal, we will be able to process human breast tissue samples rapidly and develop preliminary data on the relative levels of methylated and nonmethylated CE in breast tissue from women with and without breast cancer.

ABBREVIATIONS

CE, catechol estrogens; CE-Q, catechol estrogen quinone(s), COMT, catechol-O-methyl-transferase(s); E_1, estrone; E_2, 17β-estradiol; E_1-3,4-Q, estrone-3,4-quinone; GC/MS, gas chromatography/mass spectrometry; 4-OHE_1, 4-hydroxyestrone; 4-OHE_2, 4-hydroxyestradiol; PAH, polycyclic aromatic hydrocarbon(s); SIM, selected ion monitoring; TMS, trimethylsilyl.

Figure 3.HPLC of CE Metabolites with Electrochemical Detection

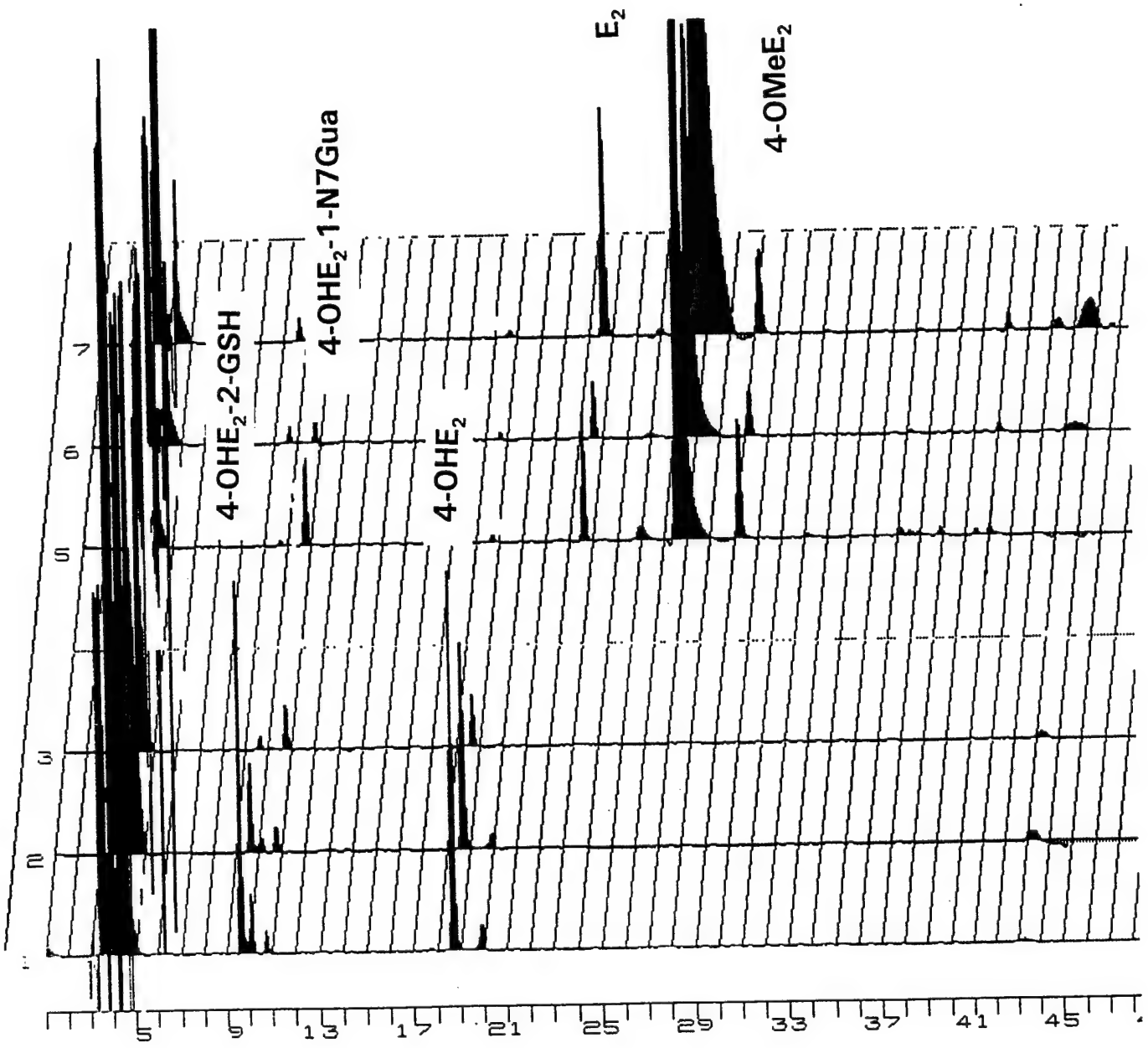


Figure 4.HPLC of CE Adduct Standards Alone

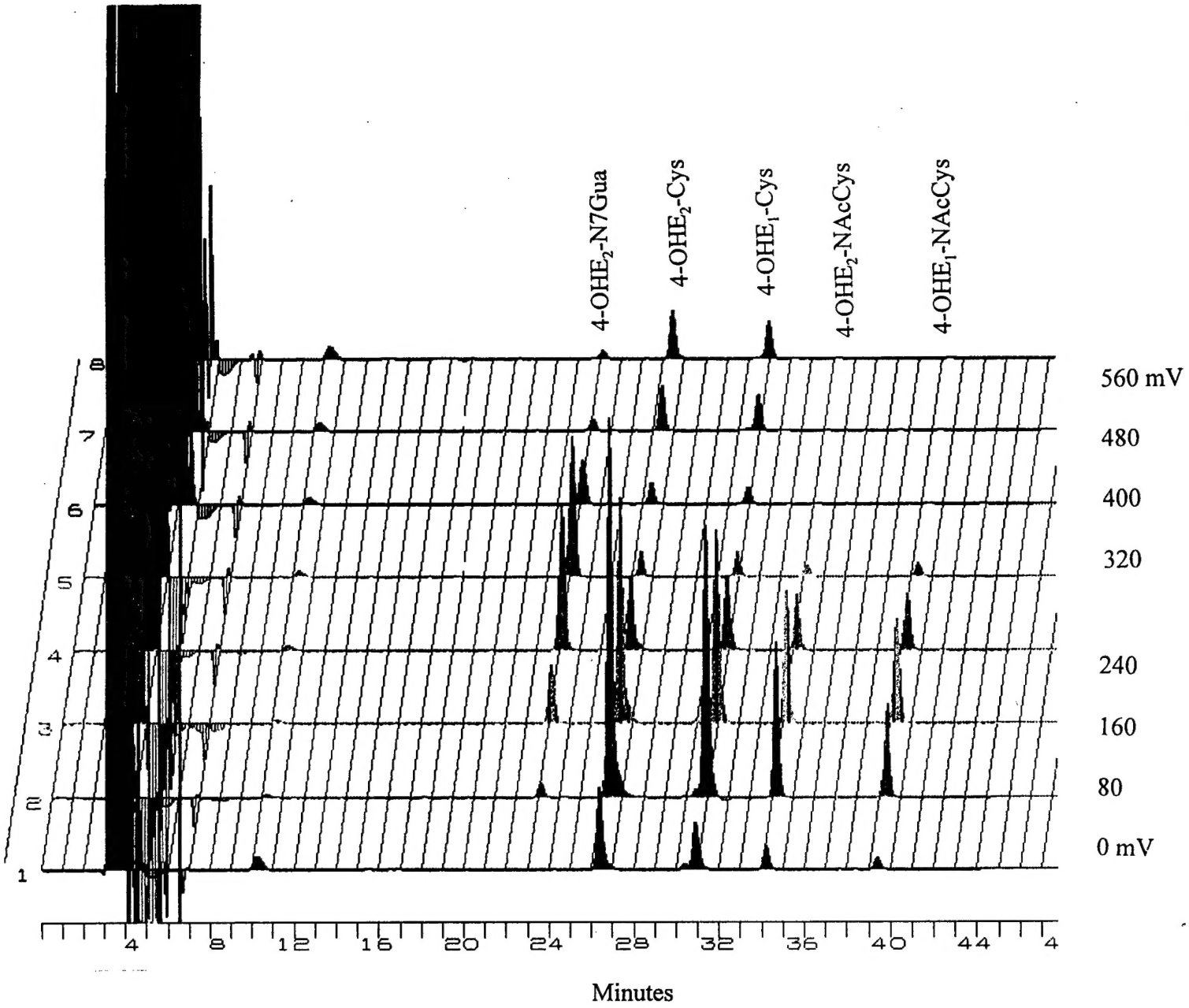
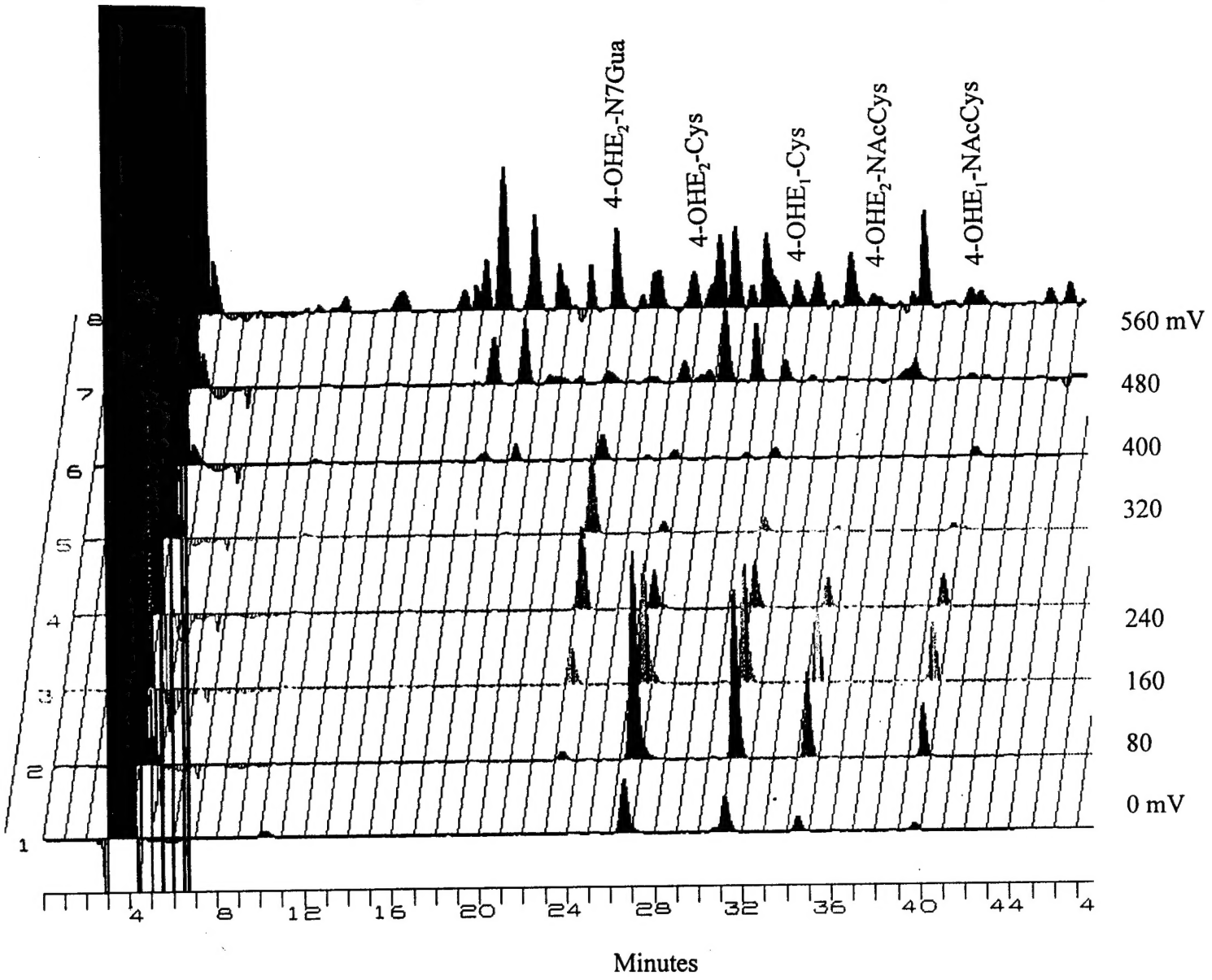


Figure 5.HPLC of Spiked Hamster Kidney with Electrochemical Detection



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